c-CBL E3 Ubiquitin Ligase Is Overexpressed in Cutaneous T-Cell Lymphoma: Its Inhibition Promotes Activation-Induced Cell Death

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Mycosis fungoides and Sézary syndrome are two major forms of cutaneous T–cell lymphoma (CTCL) characterized by resistance to apoptosis. A central pathway for T-cell apoptosis is activation-induced cell death, which is triggered through the T-cell receptor (TCR). This results in upregulation of FAS ligand (FASL) and subsequent apoptosis through the FAS death receptor pathway. It has been known for more than a decade that TCR signaling is defective in CTCL; however, the underlying mechanism has not been apparent. In this report, we show that the E3 ubiquitin ligase, c-CBL, is overexpressed in CTCL and that its knockdown overcomes defective TCR signaling, resulting in phosphorylation of PLC-g1, calcium influx, ROS generation, upregulation of FASL, and extrinsic pathway apoptosis in CTCL cells expressing adequate FAS. In CTCL cells with suboptimal FAS expression, FAS can be upregulated epigenetically by derepression of the FAS promoter using methotrexate, which we showed previously has activity as a DNA methylation inhibitor. Using these combined strategies, FAS-low as well as FAS-high CTCL cells can be killed effectively.

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INTRODUCTION

In this study, we use the term CTCL to refer specifically to mycosis fungoides (MF)/Sézary syndrome (SS) (Olsen *et al.*, 2011). On the basis of a variety of phenotypic, genetic, and functional investigations, there is ample evidence that cutaneous T-cell lymphoma (CTCL) is characterized by resistance to apoptosis (Meech *et al.*, 2001; Braun *et al.*, 2007; Contassot *et al.*, 2008; Klemke *et al.*, 2009; Wu *et al.*, 2009; Wu and Wood., 2011; Stutz *et al.*, 2012; Wu and Wood., 2014). CTCL cells often express only low levels of extrinsic apoptotic pathway death receptors such as FAS and are less responsive to apoptotic triggers compared with normal T cells. Activation-induced cell death (AICD) is a key pathway for apoptosis among CD4+/CD45RO+ memory T cells, the subset from which both MF (effector memory differentiation) and SS (central memory differentiation) are derived. Following short-term antigenic T-cell receptor (TCR) stimulation of normal CD4+ T cells, FAS is upregulated but FAS ligand (FASL) is not. The activated T cells are deleted by a passive form of intrinsic (the mitochondrial pathway) apoptosis triggered by IL-2 withdrawal following antigen clearance. In contrast, chronic TCR stimulation normally results in upregulation of both FAS and FASL with subsequent AICD involving the extrinsic FAS death receptor pathway. For several years, it has been recognized that TCR signaling is defective in CTCL such that AICD is impeded (Fargnoli *et al.*, 1997; Meech *et al.*, 2001; Klemke *et al.*, 2009). It has been observed that TCR-associated Y kinases are not properly activated upon TCR engagement and that downstream signaling eventuating in FASL upregulation does not occur. Nevertheless, an underlying mechanism for these defects has not been elucidated.

One factor that regulates TCR signaling is c-CBL, a member of the Casitas B-lineage Lymphoma protein family and a ring-type E3 ubiquitin ligase that dampens TCR function by interacting with TCR-associated Y kinases and promoting their degradation (Schmidt and Dikic, 2005; Swaminathan and Tsygankov, 2006; Loeser and Penninger, 2007; Paolino and Penninger, 2010; Qiao *et al.*, 2013). The TCR is expressed on the cell surface in association with CD3 proteins to form the TCR/CD3 complex. During AICD, engagement of TCR/CD3 normally leads to activation of proximate Y kinases and a subsequent downstream cascade involving phosphorylation of phospholipase C gamma-1 (PLC-g1), calcium mobilization, generation of reactive oxygen species (ROS), and FASL upregulation (Klemke *et al.*, 2009).

In this report, we show that c-CBL is overexpressed in CTCL and that its knockdown restores signaling through PLC-g1, leading to upregulation of FASL and apoptosis in CTCL cells that express adequate FAS. In CTCL cells with low FAS expression (a common MF/SS phenotype), FAS can be upregulated

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Abbreviations: AICD, activation-induced cell death; CTCL, cutaneous T–cell lymphoma; FASL, FAS ligand; MF, mycosis fungoides; MTX, methotrexate; PLC-g1, phospholipase C gamma-1; ROS, reactive oxygen species; siRNA, short interfering RNA; SS, Sézary syndrome; TCR, T-cell receptor

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epigenetically by derepression of the FAS promoter using methotrexate (MTX). We showed previously that in addition to its role as an S-phase inhibitor that blocks purine synthesis, MTX has activity as a DNA methylation inhibitor by blocking the synthesis of S-adenosylmethionine, the principal methyl donor for DNA methyltransferases (Wu and Wood, 2011). Using these combined strategies to modulate both FAS and FASL, FAS-low as well as FAS-high CTCL cells can be killed effectively.

RESULTS

c-CBL protein and transcript are overexpressed in CTCL

In order to assess cellular levels of c-CBL protein and messenger RNA, we analyzed CTCL cells using immunoblotting and quantitative real-time reverse-transcriptase–PCR (QRT-PCR), respectively. As shown in Figure 1a and b and Supplementary Figure 1a online, compared with normal CD4+ T cells in blood, baseline c-CBL protein and transcript were increased more than 3-fold in all five CTCL lines derived from patients with MF (MyLa, HH) or SS (SeAx, Hut-78, SZ4). Among leukemic cells from SS patients, c-CBL protein and transcript were increased more than 3-fold in 10/14 and 7/10 cases, respectively, with good correlation between individual protein and transcript levels. Lesional skin biopsies (10 patch/plaque MF, 3 tumor MF, 2 SS) were assessed by quantitative multispectral image analysis of immunoperoxidase-stained paraffin sections. Relative to reactive tonsil controls, c-CBL expression was 3-fold greater in the CTCL specimens, regardless of lesion type (Supplementary Figure 1b and c online).

c-CBL knockdown induces upregulation of FASL

Given the frequently high expression of c-CBL in CTCL, we next explored the effects of reducing c-CBL on the expression of FASL. As shown in Figure 1c, we were able to significantly decrease c-CBL protein expression in CTCL lines using short interfering RNA (siRNA) technology. As shown by flow cytometry in Figure 1d, this resulted in a significant increase in FASL expression by CTCL lines and leukemic cells from two SS patients (P1 and P2).

c-CBL knockdown induces apoptosis in CTCL cells that is enhanced by MTX

We determined the impact of c-CBL knockdown–induced FASL upregulation on apoptosis in CTCL lines and leukemic tumor cells from two SS patients using Annexin V/propidium iodide (PI) flow cytometry. Controls included isotype antibody, no treatment, and nonsense siRNA. As shown in Figure 2a, Annexin V–positive apoptotic cells (the two right-hand quadrants in each plot) increased markedly from the 3 to 9% range with nonsense siRNA to the 37–98% range with c-CBL siRNA.

MTX upregulates FAS expression epigenetically by inhibiting promoter methylation and derepressing FAS expression (Wu and Wood, 2011). As shown in Figure 2a, when MTX was combined with c-CBL siRNA knockdown, apoptosis was increased to the 81–99% range. The impact of c-CBL siRNA alone versus MTX plus c-CBL siRNA was less in MyLa (97 vs. 99%) and Hut-78 (98 vs. 99%) compared with HH (63 vs. 89%) and SZ4 (57 vs. 99%) because MyLa and Hut-78 have lower promoter methylation and higher baseline FAS expression compared with HH and SZ4 (Wu *et al.*, 2009; Wu *et al.*, 2011; Wu and Wood, 2011). In regard to potential clinical relevance, the impact of combination therapy was >2-fold in the SS leukemic cells in which apoptosis increased from 37 to 81% (P1) and from 41 to 98% (P2).

We determined the effect of MTX on c-CBL expression by QRT-PCR and could detect no significant difference in c-CBL

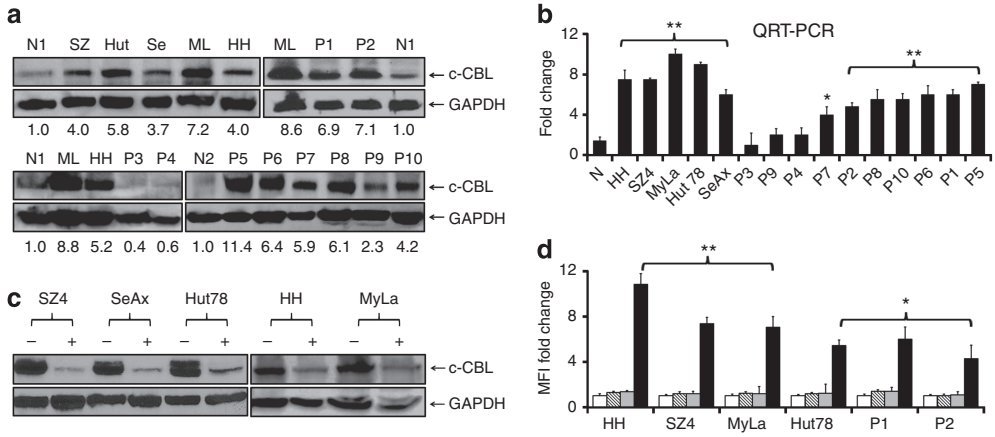

Figure 1. c-CBL is overexpressed in CTCL, and its inhibition induces FASL upregulation. (**a**) c-CBL protein. Grouped immunoblots show that, relative to the highest expression among normal CD4+ blood T cells (N1, N2), all five cutaneous T-cell lymphoma (CTCL) lines and 7/10 Sézary syndrome (SS) blood samples (P1,2,5-8,10) expressed more than 3-fold greater c-CBL protein levels, as assessed by scanning densitometry. Numbers in the immunoblots refer to fold differences in normalized c-CBL protein levels relative to control CD4+ T cells. MyLa (ML), Hut-78 (Hut), HH (H), SZ4 (SZ), and SeAx (Se). GAPDH is the loading control. (**b**) c-CBL messenger RNA. Quantitative real-time reverse-transcriptase–PCR shows that, relative to normal CD4+ blood T cells pooled from three donors (N), all five CTCL lines and the same 7/10 SS blood samples (P1,2,5-8,10) expressed more than 3-fold greater c-CBL transcript levels. *$P<0.05$; **$P<0.01$ in triplicate samples. (**c**) Grouped immunoblots show effective knockdown of c-CBL in CTCL lines using short interfering RNA (siRNA) (+) relative to nonspecific siRNA (−). GAPDH is the loading control. (**d**) Flow cytometric histogram of mean fluorescence intensities (MFIs) shows marked upregulation of FASL expression (*y*-axis) induced by c-CBL knockdown in CTCL lines and SS leukemic blood samples (P1, P2). Bars represent isotype control (white), no treatment (striped), nonsense siRNA (NS siRNA; gray), and c-CBL siRNA (black). *$P<0.05$; **$P<0.01$ for c-CBL siRNA relative to NS siRNA controls in triplicate samples.

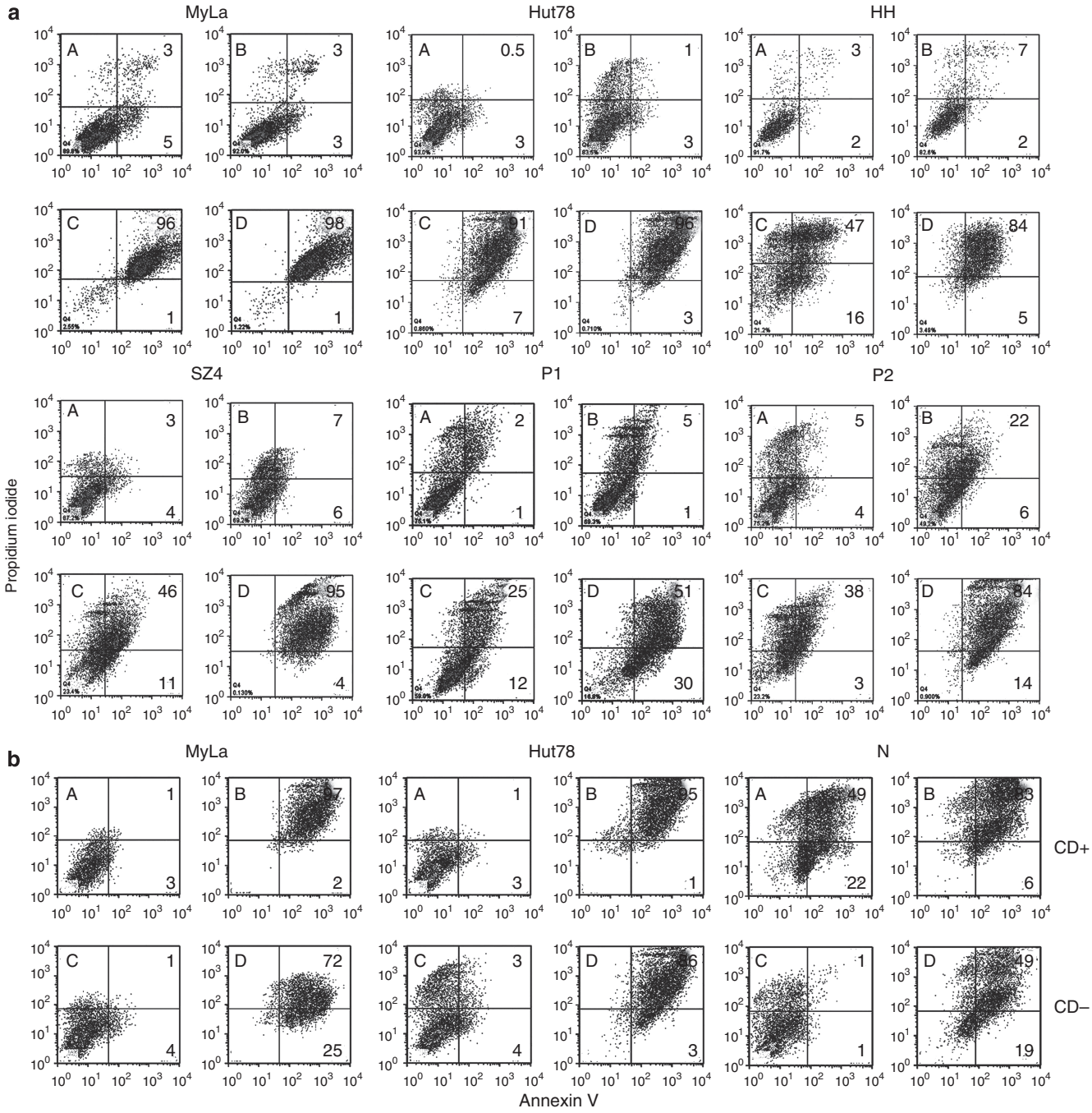

Figure 2. c-CBL inhibition induces apoptosis in CTCL, and MTX enhances this effect. (**a**) Annexin V/propidium iodide flow cytometric dot plots show marked induction of apoptosis (right two quadrants of each panel) after c-CBL knockdown. In cutaneous T-cell lymphoma (CTCL) lines (MyLa, Hut-78), which express high baseline FAS death receptor, the effect is maximal with c-CBL knockdown alone. In the other samples, treatment with methotrexate (MTX) (which upregulates low baseline FAS death receptor expression) enhances the effect of c-CBL knockdown in CTCL lines (HH, SZ4) and Sézary syndrome leukemic blood samples (P1, P2). (A) Nonsense short interfering RNA (NS siRNA), (B) MTX, (C) c-CBL siRNA, and (D) MTX plus c-CBL siRNA. Results are representative of triplicate experiments. (**b**) MyLa and Hut-78 CTCL lines show marked apoptosis after c-CBL siRNA knockdown regardless of whether cells are treated with anti-CD3/CD28 antibodies (CD+ vs. CD−) but are insensitive to antibody treatment alone. In contrast, normal CD4+ blood T cells (N) are sensitive to antibody treatment alone as well as c-CBL knockdown. (A) and (C) NS siRNA and (B) and (D) c-CBL siRNA. Results are representative of duplicate experiments.

transcript levels among five CTCL cell lines or three SS leukemic blood samples. These findings were confirmed by only minimal reductions in c-CBL protein levels detected by immunoblotting in three CTCL lines (Supplementary Figure 2 online). These data are consistent with our other evidence that MTX enhances apoptosis induced by c-CBL knockdown mainly by increasing FAS, which is then available to interact with the increased FASL resulting from c-CBL knockdown.

Impact of antibody pretreatment on c-CBL knockdown in CTCL and normal T cells

We determined whether treatment with anti-CD3 and anti-CD28 antibodies was required to induce apoptosis in FAS-high CTCL lines and normal CD4+ T cells subjected to c-CBL siRNA knockdown. Representative findings are shown in Figure 2b. CTCL cells were resistant to CD3/CD28 stimulation (3–4% apoptosis with nonsense siRNA control), whereas normal T cells were much more sensitive (71% apoptosis). In contrast, CTCL cells were more sensitive to c-CBL siRNA knockdown in the absence of CD3/CD28 stimulation (97–98%) than were normal T cells (68%). This was also reflected in the pace of apoptosis. After 96 hours of knockdown, CTCL cells had more Annexin V/PI double-positive, late apoptotic cells in the right upper quadrant of each panel (72–86%) than did normal T cells (50%).

Apoptosis induced by c-CBL knockdown is mediated by the FAS pathway

Given the fact that c-CBL knockdown significantly increased FASL expression (Figure 1d), we suspected that the associated increase in apoptosis (Figure 2) was due to extrinsic pathway apoptosis. As shown in Figure 3a, cleaved caspase 8 was significantly increased in multiple CTCL lines and SS leukemic cells as assessed by flow cytometry. Simultaneous measurement of cleaved caspase 9 using flow cytometry showed minimal or no increases (data not shown). These caspase 8 and 9 findings were confirmed by western blotting in MyLa and HH (Figure 3b). This is consistent with activation of the extrinsic rather than intrinsic (mitochondrial) apoptotic pathway (Stutz *et al.*, 2012). Several families of death receptors (e.g. FAS, TRAIL, tumor necrosis factor) can contribute to the generation of cleaved caspase 8 via the extrinsic apoptotic pathway. To determine the specific role of the FAS pathway in apoptosis induced by c-CBL knockdown and MTX, we tested the SeAx CTCL line. SeAx is FAS null because it lacks FAS genes (Wu *et al.*, 2011); yet, it does express TRAIL death receptors (Braun *et al.*, 2007). As shown in Figure 3c, FAS expression remained undetectable, and apoptosis was low (1–10%) under all conditions, despite a marked increase in FASL expression. These findings indicate that c-CBL knockdown is not cytotoxic generally and that the marked extrinsic pathway apoptosis it typically induces in CTCL cells in association with FASL upregulation probably requires expression of the FAS death receptor. This is also supported by our prior studies showing that when SeAx cells are transfected with a FAS expression vector they are readily killed by exogenous FASL (Wu *et al.*, 2009).

c-CBL knockdown enhances signaling downstream of the TCR that results in FASL upregulation

There are several known steps in the signaling cascade that link TCR engagement to FASL upregulation. These include phosphorylation of PLC-g1 at Tyr783, calcium ion influx, and generation of ROS. We used immunoblotting and flow cytometry to determine the effects of c-CBL knockdown on these parameters. As shown in Figure 3d, siRNA knockdown of c-CBL increased PLC-g1 phosphorylation in all four CTCL lines tested. This increase was confirmed by western blotting in these four cell lines and primary tumor blood samples from three SS patients (Figure 3e). The phospho-PLC-g1/total PLCg1ratio increased by 2–8-fold (median 4-fold). Similarly, induction of calcium ion influx (Figure 3f) and ROS (Figure 3g) also occurred in all four CTCL lines and tumor cells from all three SS cases tested, as assessed by Fluo-4-AM flow cytometry and 2′,7′-dichlorofluorescein diacetate (DCFDA) flow cytometry, respectively.

DISCUSSION

FAS expression in T cells is inducible by a wide variety of stimuli including MTX (Wu and Wood, 2011); however, expression of FASL is tightly controlled (Meech *et al.*, 2001), and, as shown in this report and elsewhere, CTCL cells generally express only very low levels of it (Wu *et al.*, 2009; Stutz *et al.*, 2012). Therefore, our current findings provide an approach for the upregulation of this missing trigger for the FAS apoptotic pathway. In this study, we showed that the E3 ubiquitin ligase, c-CBL, is often overexpressed in CTCL at both the protein and messenger RNA levels. This suggests a transcriptional regulatory defect, although the specific etiology of c-CBL dysregulation remains to be determined. Known mutations of CBL proteins are believed to reduce rather than enhance the ubiquitin-mediated downregulation of activated protein Y kinases. These kinases are then available to drive cell proliferation and promote neoplastic transformation (Saito *et al.*, 2012). The distribution of germline and somatic mutations are similar and cluster in the linker or ring finger domains. Germline and/or somatic mutations have been reported in chronic myelomonocytic, juvenile myelomonocytic, and acute lymphoblastic leukemias as well as in lung cancers (Lo *et al.*, 2011). CBL mutations can activate the RAS pathway. Patients with germline CBL mutations have been grouped together with those diagnosed as Noonan syndrome and related disorders under the umbrella of so-called ''RASopathies''.

Consistent with the known function of c-CBL in the degradation of receptor-associated Y kinases (Loeser and Penninger, 2007; Paolino and Penninger, 2010; Qiao *et al.*, 2013), we considered the possibility that some neoplasms might exhibit enhanced rather than diminished c-CBL function. Specifically, we postulated that c-CBL hyperactivity might help explain the long-recognized defective TCR signaling characteristic of CTCL cells (Fargnoli *et al.*, 1997; Meech *et al.*, 2001; Klemke *et al.*, 2009). Furthermore, given the importance of intact TCR signal transduction to the induction of FASL during AICD, we also postulated that the c-CBL abnormalities might have a central role in the well-known resistance of CTCL cells to apoptosis.

Our data support these hypotheses as depicted in Figure 3h. Knockdown of c-CBL using siRNA technology resulted in major increases in FASL expression among all CTCL cells tested. Several hallmarks of TCR-mediated signal transduction that lead to FASL upregulation were also enhanced. These included PLC-g1 phosphorylation, calcium ion influx, and generation of ROS. This was associated functionally with the restoration of apoptosis in CTCL cells with adequate FAS

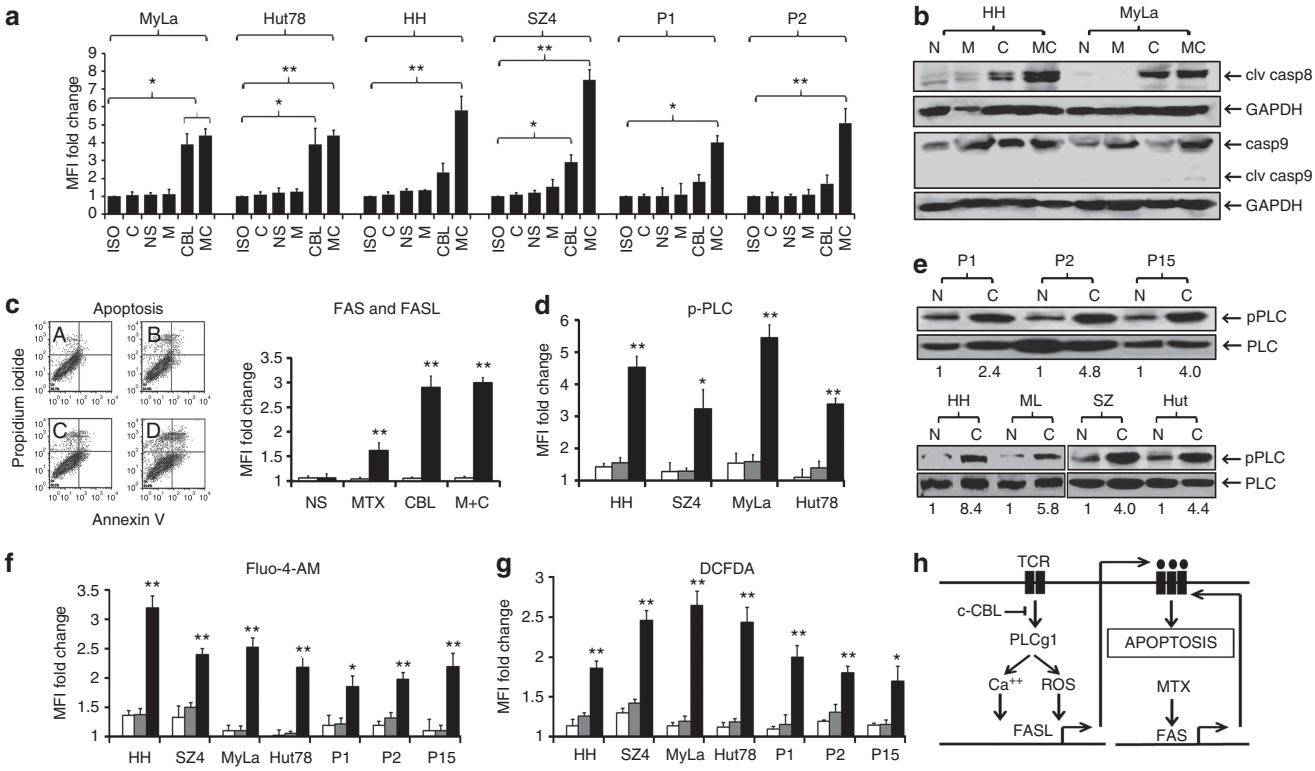

Figure 3. c-CBL inhibition in CTCL induces FAS pathway apoptosis and downstream TCR signaling. (**a**) Flow cytometric histogram of mean fluorescence intensities (MFIs) shows marked upregulation of cleaved caspase 8 expression (*y*-axis) induced by c-CBL knockdown in cutaneous T-cell lymphoma (CTCL) lines and Sézary syndrome (SS) leukemic blood samples (P1, P2). Bars represent isotype control (Iso), no treatment (C), NS siRNA (NS), MTX (M), c-CBL siRNA (CBL), and MTX plus c-CBL siRNA (MC). Analogous to the induction of apoptosis (Figure 2), samples with suboptimal baseline FAS death receptor expression (HH, SZ4, P1 and P2) showed enhanced cleavage of caspase 8 when MTX was combined with c-CBL knockdown. $*P<0.05$; $**P<0.01$ relative to NS siRNA controls in triplicate samples. (**b**) Grouped immunoblots show marked increase in cleaved caspase 8 but minimal change in cleaved caspase 9 under similar conditions to those described in **a**. NS siRNA (N), MTX (M), c-CBL siRNA (C), and MTX plus c-CBL siRNA (MC). (**c**) (Left) Annexin V/propidium iodide flow cytometric dot plots show no induction of apoptosis (right two quadrants of each panel) after c-CBL knockdown in the SeAx CTCL line, which lacks the gene for the FAS death receptor. (A) NS siRNA, (B) MTX, (C) c-CBL siRNA, and (D) MTX plus c-CBL siRNA. Results are representative of triplicate experiments. (Right) Flow cytometric histogram of MFIs shows marked upregulation of FASL (black bars) but not FAS (white bars) induced by c-CBL knockdown in CTCL line, SeAx. Histogram shows fold change compared with the ''no treatment'' control, which is set as 1. FAS levels were always null; hence, the white bars are at the *x*-axis but are shown slightly larger to be visible. NS siRNA (NS), MTX (MTX), c-CBL siRNA (CBL), and MTX plus c-CBL siRNA (M + C). $**P<0.01$ relative to NS siRNA controls in triplicate samples. (**d**) Flow cytometric histogram of MFIs shows marked upregulation of phospho-PLC-g1 induced by c-CBL knockdown. Fold change is shown with the isotype control value set as 1. Bars show no treatment (white), NS siRNA (gray), and c-CBL siRNA (black). $*P<0.05$; $**P<0.01$ relative to NS siRNA controls in triplicate samples. (**e**) Grouped immunoblots show 2–8-fold increased phospho-PLC-g1/total PLC-g1 ratios (numbers) after c-CBL siRNA knockdown (C) relative to NS siRNA control (N) set as 1. (**e–g**) Samples include four CTCL lines (HH, MyLa, SZ4, and Hut-78) and primary tumor cells from three SS patients (P1, P2, and P15). (**f**) Flow cytometric histogram of Fluo-4-AM MFIs shows that calcium flux is enhanced by c-CBL knockdown. Fold change is shown with the isotype control value set as 1. Bars show no treatment (white), NS siRNA (gray), and c-CBL siRNA (black). $**P<0.01$ relative to NS siRNA controls in triplicate samples. (**g**) Flow cytometric histogram of DCFDA MFIs shows that ROS generation is enhanced by c-CBL knockdown. Fold change is shown with the isotype control value set as 1. Bars show no treatment (white), NS siRNA (gray), and c-CBL siRNA (black). $**P<0.01$ relative to NS siRNA controls in triplicate samples. (**h**) In CTCL, the TCR signaling cascade that normally leads to FASL upregulation is blocked by high c-CBL levels. When c-CBL is inhibited, signaling is restored and FASL increases. This leads to apoptosis if there is adequate FAS death receptor expression. If FAS is low, then MTX can be used to derepress the FAS promoter, leading to FAS upregulation and subsequent apoptosis. With this approach, both FAS-high and FAS-low CTCL cells can be killed. CTCL; cutaneous T-cell lymphoma; DCFDA, 2′,7′-dichlorofluorescein diacetate; MTX, methotrexate; NS siRNA, nonsense short interfering RNA; ROS; reactive oxygen species; TCR, T-cell receptor.

expression. Among CTCL cells with suboptimal FAS expression, MTX augmented apoptosis. We demonstrated previously that MTX enhances FAS expression in CTCL cell lines and leukemic cells by inhibiting methylation of the FAS promoter and thereby derepressing gene expression (Wu and Wood, 2011). This mechanism of action for MTX appears to result from inhibition of the synthesis of S-adenosylmethionine, the main methyl donor used by DNA methyltransferases. Therefore, either alone or in combination with MTX, c-CBL knockdown was able to overcome apoptotic resistance in all CTCL cells tested, regardless of their baseline FAS expression level.

Our experiments showed that treatment with anti-CD3 and anti-CD28 antibodies is not required for c-CBL knockdown to induce apoptosis in CTCL cells. Furthermore, normal T cells were less sensitive to the pro-apoptotic effects of c-CBL knockdown than were CTCL cells, suggesting relative tumor selectivity of this approach. At present, there are no

well-characterized small molecule inhibitors specific for c-CBL. It is possible that agents affecting other E3 ubiquitin ligases or the proteasome pathway more broadly might affect the function of c-CBL. Existing inhibitors such as thalidomide, lenalidomide, and bortezomib have already shown some efficacy in CTCL (Jain *et al.*, 2012; Querfeld *et al.*, 2014), and additional agents are being developed (Lydeard and Harper, 2010). Our current findings might help explain the mechanism of action of these drugs in CTCL. Histone deacetylase inhibitors like tenovin-1 can also indirectly affect the function of some E3 ubiquitin ligases (Chen *et al.*, 2007). Our data suggest that an appropriate small molecule inhibitor of c-CBL, either alone or in combination with MTX, might provide an effective approach to the treatment of CTCL. Because MTX's ability to inhibit DNA methylation is shared by other folate antagonists, currently approved drugs such as pralatrexate and pemetrexed might also be of value in this therapeutic context. Our prior work also showed that interferon-alpha increased FAS expression in CTCL cells by a STAT-1-mediated mechanism distinct from the demethylating action of MTX (Wu *et al.*, 2011) and that their combined effects on FAS-mediated apoptotic sensitivity were synergistic (Wu and Wood, 2011). Therefore, addition of this agent to a multidrug regimen might also prove beneficial in some cases.

In a larger context, our c-CBL knockdown data provide proof-of-principle that, whether by targeting c-CBL or other mechanisms, upregulation of FASL is an effective strategy for killing CTCL tumor cells driven by various proliferative signals such as the well-documented constitutive activation of NF-κB (Sors *et al.*, 2008). The physiologic balance between apoptosis and proliferation in normal T cells is disrupted in CTCL by constitutive overactivity of both c-CBL and proliferative stimulation. This leads to blocked AICD and unchecked proliferation. However, when c-CBL is blocked, FASL is upregulated. AICD can then occur, despite constitutively active proliferative drivers.

Aside from c-CBL, there are several alternative approaches that might be exploited to upregulate FASL. It has been reported that IL-2 plus PKC activators (PMA or bryostatin) that act downstream of the TCR can induce leukemic CTCL cells to express FASL and undergo apoptosis *ex-vivo* (Meech *et al.*, 2001). Similarly, UV therapy and photodynamic therapy might also be able to overcome defective TCR signaling by inducing ROS and upregulating FASL through the JNK–Jun pathway. This mechanism for FASL upregulation has been reported in squamous cell carcinomas (Ahmad *et al.*, 2000; Ali *et al.*, 2002; Furre *et al.*, 2006). However, AP1 transcription factors containing c-Jun repress FAS expression. Therefore, concomitant therapy with MTX or other DNA methylation inhibitors might be important for ensuring the presence of sufficient FAS to interact with FASL and trigger apoptosis. In fact, our recent studies show that PDT induces FAS ligand in CTCL and that MTX + PDT induces both FAS and FAS ligand, resulting in significantly enhanced apoptosis relative to PDT alone (Salva *et al.*, 2014). Finally, it has been shown that nonsteroidal anti-inflammatory agents such as diclofenac can promote extrinsic pathway apoptosis by decreasing the inhibitory factor c-FLIP (Braun *et al.*, 2012).

MATERIALS AND METHODS

CTCL cell lines, SS blood specimens, and MF/SS skin lesions

Human CTCL lines derived from patients with MF (MyLa, HH) or SS (SZ4, Hut-78, SeAx) were studied. All have been described previously (Wu *et al.*, 2009; Wu and Wood, 2011). Cells were cultured under standard conditions and harvested by centrifugation.

All clinical samples were obtained with institutional review board approval. All patients gave written informed consent, and all protocols adhered to the Declaration of Helsinki principles. BMCs were obtained from 15 SS patients (P1-P15). This included 6 men and 9 women: 2 stage IIIB, 12 stage IVA, and 1 stage IVB. In all cases, the majority of cells were tumor cells as assessed by V-beta TCR antibodies, CD4 + 26 − phenotype, and/or Sézary preparations (11 in the 86–99% range; 4 in the 61–71% range). Lesional skin biopsies were obtained from 8 men and 7 women: 10 patch/plaque MF (2 stage IA, 5 stage IB, and 3 stage IIB), 3 tumor MF (all stage IIB), and 2 SS (stages IVA and IVB). Samples were obtained prior to or 1 month off therapy. Multispectral image analysis using the Nuance computerized microscope system was employed for quantitative measurement of *in-situ* c-CBL protein expression in these biopsies as described previously by us (Wu *et al.*, 2014).

In some experiments, cells were stimulated with a combination of monoclonal antibodies directed against CD3 (OKT3; eBioscience; San Diego, CA) and CD28 (CD28.2; BD Biosciences; San Jose, CA) prior to other interventions. Cell culture plates were pre-coated with anti-CD3 antibody (30 μg ml^{-1}) for 24 hours at 4 °C. Cells then were stimulated with plate-bound anti-CD3, soluble anti-CD28 (3 μg ml^{-1}), and goat anti-mouse (2.5 μg ml^{-1}) antibodies (BD Biosciences) for 24 hours. In some experiments, cells were treated with MTX, which was obtained from MP Biomedicals (Santa Ana, CA), dissolved in DMSO, and diluted to 10 μM in culture medium.

Flow cytometry

Expression of FAS, FASL, phospho-PLC-g1, and cleaved caspase 8 was assessed by flow cytometry. Cells were stained with FITC-conjugated antibody against FAS/CD95 (DX2), biotin-conjugated (NOK-1) or unconjugated (G247-4) antibody against FAS ligand/CD178 (BD Biosciences), anti-phospho-PLC-g1 (Tyr783), and anti-cleaved caspase-8 (18C8) (Cell Signaling, Beverly, MA). Isotype-matched mAbs of irrelevant specificity were used as negative controls. Briefly, 2×10^5 lymphoid cells were washed twice with phosphate-buffered saline (PBS) and blocked with 1:10 normal goat serum in PBS for 20 minutes. For phospho-PLC-g1 and cleaved caspase 8 detection, cells were fixed by Cytofix Buffer and permeablized by Perm Buffer III (BD Biosciences). Cells were then immunostained for 30 minutes at room temperature and washed twice with PBS. If needed, secondary staining with FITC-conjugated avidin or goat anti-mouse IgG (BD Biosciences) was then performed, followed by further washing. Cells were then resuspended in FACS buffer (2% BSA in PBS) and analyzed with a FACSCalibur bench top flow cytometer (BD Biosciences). Data analysis was performed using FlowJo software (Treestar, Ashland, OR).

For Annexin V/PI apoptosis analysis, control and treated cells were collected, washed with PBS and then resuspended in 100 μl binding buffer (0.1 M Hepes, pH 7.4; 1.4 M NaCl; 25 mM $CaCl_2$), and stained with FITC-conjugated Annexin V and PI (BD Biosciences) as per vendor's guidelines. After staining, flow cytometric analysis was performed as described above. Annexin V single-positive cells were

interpreted as early apoptotic cells (right lower quadrant of dot plots). Annexin V/PI double-positive cells were interpreted as late apoptotic cells (right upper quadrant of dot plots).

Calcium mobilization and ROS assays

To assess calcium ion flux, cells were stained with Fluo-4-AM dye, and, to assess the generation of ROS, cells were stained with DCFDA and subjected to flow cytometric analysis as described above. For Fluo-4-AM staining, cells were washed and resuspended in PBS, and Fluo-4-AM was added to a final concentration of 1 μM. Cells then were incubated in room temperature for 1 hour. For DCFDA staining, DCFDA was directly added to 2 ml of cell culture to the final concentration of 5 μM and incubated at 37 °C for 30 minutes. Stained cells were washed and analyzed by flow cytometry.

Immunoblotting

Following treatments, cells were washed with ice-cold PBS and lysed with RIPA buffer. For immunoblot analysis, 30 μg of each protein sample was subjected to SDS-PAGE and transferred onto a nitrocellulose membrane. Blots were exposed to anti-c-CBL (D4E10), cleaved caspase 8 (18C8), caspase 9 (C9), PLC-g1 (D9H10) and phospho-PLC-g1 (Tyr 783) primary antibodies (Cell Signaling; Beverly, MA), and horseradish peroxidase-conjugated appropriate secondary antibodies (Cell Signaling; Danvers, MA), followed by enhanced chemiluminescent detection (Thermo Fisher Scientific, Rockford, IL) using the Fotodyne digital imaging system (Fotodyne, Hartland, WI). Loading control antibody was anti-GAPDH (D16H11; Cell Signaling; Beverly, MA). Numbers in immunoblot lanes represent scanning densitometry relative to controls.

Quantitative real-time reverse-transcriptase–PCR

RNA was isolated with Trizol reagent (Life Technologies, Grand Island, NY), treated with DNase (Promega, Madison, WI), and first-strand complementary DNA created with M-MLV reverse transcriptase (Promega) according to the vendor's protocol. QRT-PCR was performed with SYBR Green PCR Master Mix (Life Technologies). Relative c-CBL messenger RNA expression was calculated using the $\Delta\Delta C_T$ method with GAPDH as an endogenous control. The primer sequences and conditions for QRT-PCR are shown below:

	Sequence (5′→3′)	Length	*Tm*	Location
Fw c-CBL primer	GTGATCCCTGGACAGGAAGA	20	60.0	1959–1979
Rev c-CBL primer	CATTGGCAGATGAGGAAGGT	20	62.4	2156–2176
Fw GAPDH primer	TGTGGGCATCAATGGATTTGG	21	60.9	231–251
Rev GAPDH primer	ACACCATGTATTCCGGGTCAAT	22	61.4	346–325

Abbreviations: Fw, forward; Rev, reverse.

c-CBL knockdown

Knockdown of c-CBL was performed using siRNA technology. Four pooled siRNA oligos targeting c-CBL (#1027416) or nonspecific siRNAs (#1027280) were obtained from Qiagen (Valencia, CA) and were transfected into cells by electroporation using the Nucleofector device and the L kit (Lonza, Basel, Switzerland). Briefly, cells were cultured under normal conditions to the log-growth stage, 3×10^6 cells were centrifuged and resuspended in 100 μl of transfection solution L, and siRNA was added to a final concentration of 1 μM and transfected using pre-installed program X-001 in Nucleofector. The c-CBL siRNA sequences were as follows: 5′-CCCGCCGAACTCTCTCAGATA-3′, 5′-CCGTACTATCTTGTCAAGATA-3′, 5′-CCCATACTTCGTATTCTTAAA-3′, and 5′-CTGGCGCTAAAGAATAGCCCA-3′. Cells were cultured for 4 days prior to detection or further processing. Controls included isotype antibody, no treatment, and nonsense siRNA.

Statistics

Statistical analysis was performed using Student's *t*-test. A *P*-value < 0.05 was considered statistically significant. Error bars in histograms show standard deviations of triplicate measurements.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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